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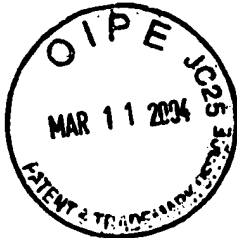
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Jose Repolles Moliner	Examiner:	D. Lukton
Serial No.:	09/912,164	Group Art Unit:	1653
Filed:	March 16, 2001	Docket:	14797
For:	S-NITROSOTHIOLS AS AGENTS FOR THE TREATMENT OF CIRCULATORY DYSFUNCTIONS		

Confirmation No. 4564

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF JOSE REPOLLES MOLINER UNDER 37 C.F.R. §1.132

Sir:

I, Jose Repolles Moliner declare and state as follows:

1. I am one of the inventors of the subject matter in the above-identified application, and I have complete knowledge of all aspects of the invention therein.
2. The present invention is directed to S-nitrosothiols as agents for the treatment of circulatory dysfunctions, especially at the cardiovascular level.
3. I have reviewed the above-identified application and I am familiar with all aspects of the invention described therein.
4. With regard to the Office Action dated September 9, 2003, I have been advised by counsel that the Examiner has rejected claims 28-37 of the present application under 35 U.S.C. §112, first paragraph alleging that the description therein does not enable one of ordinary skill in the art to make or use the invention.

5. I have been asked by counsel to show that compounds of the present invention have utility in treating disorders of the circulatory system.

6. In response, to counsel's request, I have performed the following experiments. These experiments were conducted by me or under my direct supervision and control.

7. In accordance with an aspect of the present invention, the *in vivo* antithrombotic effect of representative compounds of the invention using scientifically recognized assays was investigated. In one such assay, using the method described in paragraph 8, compounds identified in the specification as 2 and 5, viz., N-acetyl-2-amino-2-[4-S-nitrosomercapto-1-methylpiperidin)] acetic acid and N[N- γ -L-glutamyl-2-amino-2-(4-(4-S-nitrosomercapto-1-methylpiperidin)) acetyl]glycine, respectively, were tested for *in vivo* anti-thrombotic activity.

8. The method used is substantially the same as described by Kurz (Kurz K.D., et al., Thromb. Res. 1990, 60:269-280) and modified by Feuerstein (Feuerstein G.Z., et al., Arterioscler. Thromb. Vasc. Biol. 1999, 19:2554-2556). Rats were anaesthetized with sodium pentobarbital (40mg/kg, i.p.) and then placed dorsally on a heated (37°C) surgical board. Rats received an infusion of 0.3 nmol/Kg/min of compounds 2 and 5, respectively. GSNO, S-nitrosogluathione, a reference compound, was used as a control using the same procedure, it was separately administered to the rat. The left carotid artery was also isolated and a Parafilm M sheet (7 x 20 mm, American National Can) was placed under it. An electromagnetic blood flow probe (Transonic Systems Inc.,) was placed on the artery, to measure blood flow.

9. Fifteen minutes after the infusion of the compounds was started, a 6 x 8 mm filter paper patch saturated with FeCl₃ solution (70%) was placed (and not removed for the whole duration of the experiment) on the left carotid artery downstream from the flow probe to initiate

thrombosis. Blood flow was monitored for the next 35 minutes following application of the patch on the artery. At 35 minutes following application of the patch on the artery the infusion of the compounds was halted.

10. The vessel was considered occluded by the thrombus formed when no blood flow was detected (0,0 ml/min). In this model, thrombus formation usually takes place within 15 to 20 minutes in non-treated animals. An animal was considered as fully protected by treatment if a thrombus did not occlude the vessel during the period of study (30 minutes after FeCl₃ patch application).

11. The results are shown in Table 1 and are expressed as percentage of animals fully protected by the treatment.

Table 1

In vivo anti-thrombotic activity	
Compound	% of animals fully protected
Reference (GSNO)	32
2	68
5	50

12. As can be observed in Table 1, the compounds of the present invention exhibit significant *in vivo* anti-thrombotic activity.

13. I have had verified that compounds of the present invention exhibit an *in vivo* antithrombotic effect using a second scientifically recognized assay. Using the method described in paragraph 14, compounds 2 and 5, identified in the specification, as N-acetyl-2-amino-2-[4-S-nitrosomercapto-1-methylpiperidin]] acetic acid and N[N-γ-L-glutamyl-2-amino-2-(4-(4-S-

nitrosomercapto-1-methylpiperidin)) acetyl]glycine, respectively, were tested for *in vivo* anti-thrombotic activity by another assay.

14. The method used is substantially the same as described by Smith JR and White AM. Fibrin, red cell and platelet interactions in an experimental model of thrombosis. Br. J. Pharmacol., 1982; 77:029-038.

15. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and then placed dorsally on a heated (37°C) surgical board. Rats received an infusion of 0.1 $\mu\text{mol/Kg/min}$ of compound 2, 5, or GNSO. The left carotid artery and right jugular vein were isolated and fifteen minutes after the infusion of the compounds was started the artery and vein segments were connected through a polyethylene tube with a cotton wire inside. Fifteen minutes after the artery and veins segments were connected, the tube connecting both vascular segments was disconnected and the clot on the cotton wire was weighed.

16. The results are shown in Table 2 and are expressed as percentage of reduction in clot weight.

Table 2

In vivo anti-thrombotic activity	
Compound	% of clot weight reduction
Reference (GSNO)	50
2	60
5	70

17. As it can be observed by the data in Table 2, the compounds of the present invention tested exhibited a significant potent *in vivo* anti-thrombotic activity in this test.

18. In accordance with an aspect of the present invention, the *ex vivo* antithrombotic effect of representative compounds of the invention using a scientifically recognized assay was also investigated. Using the methodology described in Paragraph 19 below, compounds 2 and 5 identified in the specification as N-acetyl-2-amino-2-[4-S-nitrosomercapto-1-methylpiperidin]] acetic acid and N[N- γ -L-glutamyl-2-amino-2-(4-(4-S-nitrosomercapto-1-methylpiperidin)) acetyl]glycine, respectively, were tested for *ex vivo* antithrombotic activity.

19. The *ex vivo* bleeding time -closure time- was measured using PFA-100[®] (Dade NV, Amestford, The Netherlands). The instrument aspirates a blood sample under constant vacuum from the sample reservoir through a capillary and a microscopic aperture punctured into a membrane. The membrane is coated with collagen and epinephrine or collagen and ADP. [See, Kundu SK, Heilman EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an *in vitro* platelet function analyzer-PFA-100. Sem. Thromb. Hemost. 1995;21:106-112] The shear rate achieved at the level of the aperture is equivalent to 5000-6000 sec⁻¹. The presence of these biochemical stimuli and the high shear rates generated under standardized flow conditions result in platelet adhesion, activation and aggregation, slowly building up a stable platelet plug in the aperture. The time required to obtain full occlusion of the aperture, i.e., closure time, is reported as a measurement of the hemostatic performance of platelets in the analyzed blood sample.

20. New Zealand rabbits weighing approximately 3 Kg were used for this experiment. The rabbits were anesthetized with ketamine (40 mg/Kg) and xilazine (5 mg/Kg), then they received an infusion of the compounds 2, 5 or GSNO, at 0.1 μ mol/Kg/min for 10 min through the ear vein. At the end of the infusion, a blood sample was obtained from the carotid artery. This blood sample was used to evaluate the closure time.

21. The results are shown in Table 3 and are expressed as percentage of increase in closure time in relation to the closure time in a sample obtained before the infusion of compounds.

Table 3

In vivo antithrombotic activity	
Compound	% of increase in closure time
Reference (GSNO)	30
2	100
5	80

22. As it can be observed in Table 3, based on the result of these tests, the compounds of the present invention tested have significant *in vivo* antithrombotic activity.

23. In accordance with another aspect of the present invention, the *in vivo* hypotensive effect of representative compounds of the invention was investigated. Using the methodology described in Paragraph 24, compounds 2 and 5 identified in the specification as N-acetyl-2-amino-2-[4-S-nitrosomercapto-1-methylpiperidin]] acetic acid and N[N- γ -L-glutamyl-2-amino-2-(4-(4-S-nitrosomercapto-1-methyl-piperidin)) acetyl]glycine, respectively, were tested for *in vivo* hypotensive activity.

24. The method used is substantially the same as described by D.D. Rees, J.E. Monkhouse, D. Cambridge & S. Moncada, Br. J. Pharmacol., 1998; 124:540-546. Rats were anaesthetized with 2% isoflurane. A canular was implanted in the femoral artery and femoral vein, tunneled subcutaneously to exit at the top of the back and connected to a swivel tether system for continuous monitoring to blood pressure and drug administration, respectively. Normal physiological saline (154 mM) containing heparin (24 u/ml) was administered as a continuous infusion via the femoral artery (50 μ l/h) to maintain patency of the blood pressure

cannula line. Following recovery from surgery, blood pressure was measured over the following 24 hour period. Only animals showing a mean blood pressure within the normal range (90-110 mmHg) over this period were entered into the study.

25. Compounds 2, 5 or GSNO (0.3 μ mol/Kg/min at 1 ml/min), were administered via the femoral vein for one hour and the blood pressure was measured during the time of administration and over the following 1 hour period.

26. The results are shown in Table 4 and are expressed as decrease in blood pressure.

Table 4

<i>In vivo</i> hypotensive activity	
Compound	Decrease in blood pressure (mmHg)
Reference (GSNO)	11
2	9
5	13

27. As it can be observed in Table 4, the compounds of the present invention exhibit hypotensive activity.

28. Hypertension and thrombotic disorders are representative examples of "dysfunctions" of the circulatory system, which is a term of art understood by one of ordinary skill in the art.

29. The data herein clearly illustrate, in any opinion, to one of ordinary skill in the art that the claimed subject matter including the subject matter in Claims 28-37 exhibit *in vivo* pharmaceutical activity. Moreover, the data clearly illustrate, in my opinion, that the compounds of the present invention are useful for treating disorders of the circulatory system.

30. The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of the title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: March 9th, 2004


Jose Repolles Moliner